



**EFSA Panel on Food Contact Materials , Enzymes, Flavourings and Processing Aids (CEF); Scientific Opinion on Flavouring Group Evaluation 224 (FGE.224): Consideration of genotoxic potential for two , - unsaturated thiophenes from subgroup 5.2 of FGE.19 by EFSA.**

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## SCIENTIFIC OPINION

### **Scientific Opinion on Flavouring Group Evaluation 224 (FGE.224): Consideration of genotoxic potential for two $\alpha,\beta$ -unsaturated thiophenes from subgroup 5.2 of FGE.19 by EFSA<sup>1</sup>**

**EFSA Panel on Food Contact Materials, Enzymes,  
Flavourings and Processing Aids (CEF)<sup>2,3</sup>**

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This scientific output, published on 10 April 2013, replaces the earlier version published on 20 February 2013.<sup>4</sup>

#### **ABSTRACT**

The Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids of the European Food Safety Authority was requested to evaluate the genotoxic potential of two flavouring substances from subgroup 5.2 of FGE.19 in the Flavouring Group Evaluation 224 (FGE.224). The Flavour Industry has provided additional genotoxicity studies for one of the two substances in FGE.224, namely 5-methyl-2-thiophenecarbaldehyde [FL-no: 15.004]. The data requested by EFSA for the other substance, 3-acetyl-2,5-dimethylthiophene [FL-no: 15.024] of FGE.224 will be provided subsequently according to the Flavour Industry. Based on the new data the Panel concluded that 5-methyl-2-thiophenecarbaldehyde does not give rise to concern with respect to genotoxicity and can accordingly be evaluated using the Procedure. For the other substance in subgroup 5.2, 3-acetyl-2,5-dimethylthiophene, the requested genotoxicity data are still pending and no conclusion could be drawn in the present FGE.

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#### **KEY WORDS**

FGE.224,  $\alpha,\beta$ -unsaturated thiophenes,  $\alpha,\beta$ -unsaturated thiophenes, Subgroup 5.2, FGE.19

<sup>1</sup> On request from the European Commission, Question No EFSA-Q-2012-00678, adopted on 31 January 2013.

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<sup>4</sup> Editorial changes have been made to pages 3, 4, 5 and 6 (History of evaluation) and to tables 1, 2 and 3 (double bonds in chemical structure). The changes do not affect the overall conclusions of the scientific opinion.

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## SUMMARY

Following a request from the European Commission the Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF Panel) was asked to deliver a scientific opinion on the implications for human health of chemically defined flavouring substances used in or on foodstuffs in the Member States. In particular, the Panel was requested to consider the Joint FAO/WHO Expert Committee on Food Additives (the JECFA) evaluations of flavouring substances assessed since 2000, and to decide whether no further evaluation is necessary, as laid down in Commission Regulation (EC) No 1565/2000. These flavouring substances are listed in the Register, which was adopted by Commission Decision 1999/217/EC and its consecutive amendments.

The present Flavouring Group Evaluation 224 (FGE.224), corresponding to subgroup 5.2 of FGE.19, concerns two  $\alpha,\beta$ -unsaturated thiophenes. The two substances in the present evaluation are one  $\alpha,\beta$ -unsaturated aldehyde and one  $\alpha,\beta$ -unsaturated ketone, structures which are considered to be structural alerts for genotoxicity and the data on genotoxicity previously available for these two substances did not rule out the concern for genotoxicity.

The Panel did not find that genotoxicity data for either of the substances in subgroup 5.2 could be used for reading across to the other substance in the subgroup; therefore genotoxicity data have been requested for both substances, according to the test strategy worked out by the Panel.

The Flavour Industry has provided additional genotoxicity data for one 5-methyl-2-thiophenecarbaldehyde [FL-no: 15.004] of the two substances in FGE.224. The requested data for the other substance, 3-acetyl-2,5-dimethylthiophene [FL-no: 15.024], will be provided subsequently, according to the Flavour Industry.

Based on the data submitted the Panel concluded that 5-methyl-2-thiophenecarbaldehyde [FL-no: 15.004] does not give rise to concern with respect to genotoxicity and can accordingly be evaluated using the Procedure. For the other substance in subgroup 5.2, 3-acetyl-2,5-dimethylthiophene [FL-no: 15.024], the requested genotoxicity data are still pending and no conclusion could be drawn in the present FGE.

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**BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION**

In the 26<sup>th</sup> Plenary meeting of the AFC Panel on 27-29 November 2007, EFSA discussed the Flavouring Group Evaluation 19 (FGE.19). FGE.19 contains those flavouring substances which are  $\alpha,\beta$ -unsaturated aldehydes or ketones and their precursors which could give rise to such carbonyl substances via hydrolysis and/or oxidation. The  $\alpha,\beta$ -unsaturated aldehyde and ketone structure is considered by the Panel to be a structural alert for genotoxicity. FGE.19 was divided into subgroups. For subgroup 5.2, which contains 2 flavouring substances, EFSA concluded that there is a need for additional information before conclusions on the substances in this subgroup can be reached.

Information on flavouring substance 5-methyl-2-thiophenecarbaldehyde (FL-no: 15.004) has now been submitted by the European Flavour Association.

The Commission asks EFSA to evaluate this new information and depending on the outcome proceed to the full evaluation of this flavouring substance.

**TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION**

The European Commission requests the European Food Safety Authority to carry out a safety assessment on 5-methyl-2-thiophenecarbaldehyde [FL-no: 15.004], in accordance with Commission Regulation (EC) N° 1565/2000.

## ASSESSMENT

### 1. History of the Evaluation

Regulation (EC) N° 2232/96 of the European Parliament and the Council (EC, 1996) lays down a procedure for the establishment of a list of flavouring substances, the use of which will be authorised to the exclusion of all other substances in the EU. In application of that Regulation, a Register of flavouring substances used in or on foodstuffs in the Member States was adopted by Commission Decision 1999/217/EC (EC, 1999), as last amended by Commission Decision 2009/163/EC (EC, 2009). Each flavouring substance is attributed a FLAVIS-number (FL-number) and all substances are divided into 34 chemical groups. Substances within a group should have some metabolic and biological behaviour in common.

Substances which are listed in the Register are to be evaluated according to the evaluation programme laid down in Commission Regulation (EC) N° 1565/2000 (EC, 2000) which is broadly based on the opinion of the Scientific Committee on Food (SCF, 1999). For the submission of data by the manufacturer, deadlines have been established by Commission Regulation (EC) N° 622/2002 (EC, 2002).

The Union list of flavourings and source materials is established in Commission Regulation (EC) N° 872/2012 (EC, 2012).

Flavouring Group Evaluation 19 (FGE.19) contains 360 flavouring substances from the EU Register being  $\alpha,\beta$ -unsaturated aldehydes or ketones and precursors which could give rise to such carbonyl substances via hydrolysis and / or oxidation (EFSA, 2008a).

The  $\alpha,\beta$ -unsaturated aldehyde and ketone structures are structural alerts for genotoxicity. The Panel noted that there were limited genotoxicity data on these flavouring substances but that positive genotoxicity studies were identified for some substances in the group.

The  $\alpha,\beta$ -unsaturated carbonyls were subdivided into subgroups on the basis of structural similarity (EFSA, 2008a). In an attempt to decide which of the substances could go through the Procedure, a (quantitative) structure-activity relationship ((Q)SAR) prediction of the genotoxicity of these substances was undertaken considering a number of models (DEREKfW, TOPKAT, DTU-NFI MultiCASE Models and ISS Local Models, (Gry et al., 2007)).

The Panel noted that for most of these models internal and external validation has been performed, but considered that the outcome of these validations was not always extensive enough to appreciate the validity of the predictions of these models for the  $\alpha,\beta$ -unsaturated carbonyls. Therefore, the Panel considered it inappropriate to totally rely on (Q)SAR predictions at this point in time and decided not to take substances through the Procedure based on negative (Q)SAR predictions only.

The Panel took note of the (Q)SAR predictions by using two ISS Local Models (Benigni and Netzeva, 2007a; Benigni and Netzeva, 2007b) and four DTU-NFI MultiCASE Models (Gry et al., 2007; Nikolov et al., 2007) and the fact that there are available data on genotoxicity, *in vitro* and *in vivo*, as well as data on carcinogenicity for several substances.

Based on these data the Panel decided that 15 subgroups (1.1.1, 1.2.1, 1.2.2, 1.2.3, 2.1, 2.2, 2.3, 2.5, 3.2, 4.3, 4.5, 4.6, 5.1, 5.2 and 5.3) (EFSA, 2008a) could not be evaluated through the Procedure due to concern with respect to genotoxicity. Corresponding to these subgroups, 15 Flavouring Group Evaluations (FGEs) were established, FGE.200, 204, 205, 206, 207, 208, 209, 211, 215, 219, 221, 222, 223, 224 and 225).

For 11 subgroups the Panel decided, based on the available genotoxicity data and (Q)SAR predictions, that a further scrutiny of the data should take place before requesting additional data on genotoxicity from the Flavouring Industry. These subgroups were evaluated in FGE.201, 202, 203, 210, 212, 213,

214, 216, 217, 218 and 220. For the substances in FGE.202, 214 and 218 it was concluded that a genotoxic potential could be ruled out and accordingly these substances will be evaluated using the Procedure. For all or some of the substances in the remaining FGEs, FGE.201, 203, 210, 212, 213, 216, 217 and 220 the genotoxic potential could not be ruled out.

To ease the data retrieval of the large number of structurally related  $\alpha,\beta$ -unsaturated substances in the different subgroups for which additional data are requested, EFSA has worked out a list of representative substances for each subgroup (EFSA, 2008c). Likewise an EFSA genotoxicity expert group has worked out a test strategy to be followed in the data retrieval for these substances (EFSA, 2008b).

The Flavouring Industry has been requested to submit additional genotoxicity data according to the list of representative substances and test strategy for each subgroup.

The Flavouring Industry has now submitted additional data and the present FGE concerns the evaluation of some of these data requested on genotoxicity.

## 2. Presentation of the substances in the Flavouring Group Evaluation 224

### 2.1. Description

The present Flavouring Group Evaluation 224 (FGE.224), corresponding to subgroup 5.2 of FGE.19, concerns two  $\alpha,\beta$ -unsaturated thiophene derivatives, one  $\alpha,\beta$ -unsaturated aldehyde, 5-methyl-2-thiophenecarbaldehyde [FL-no: 15.004] and one  $\alpha,\beta$ -unsaturated ketone, 3-acetyl-2,5-dimethylthiophene [FL-no: 15.024]. The  $\alpha,\beta$ -unsaturated aldehyde and ketone structures are considered to be structural alerts for genotoxicity (EFSA, 2008a) and the data on genotoxicity previously available did not rule out this concern for genotoxicity.

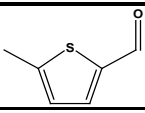
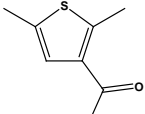
Furthermore, the Panel did not find that the chemical structure of the two substances allowed for a read across between genotoxicity data for the two substances and accordingly the Flavour Industry was requested to submit data for each of the substances in subgroup 5.2. The structures of the two substances in Subgroup 5.2 are shown in Tables 1 and 2.

The two substances have previously been evaluated by the JECFA at their 59<sup>th</sup> meeting (JECFA, 2002a, 2003). A summary of their current evaluation status by the JECFA and the outcome of this consideration is presented in Table 3.

### 2.2. Representative substances for subgroup 5.2 of FGE.19

As the Panel did not find that the chemical structure of the two substances allowed for a read across between genotoxicity data, the Flavour Industry was requested to submit genotoxicity data for each of the substances in subgroup 5.2 in accordance with the test strategy (EFSA, 2008b). The chemical structures of the two substances in subgroup 5.2 are shown in Table 1.

**Table 1:** Substances in subgroup 5.2 of FGE.19

FL-no JECFA-no	EU Register name	Structural formula	FEMA no CoE no CAS no
15.004 1050	5- Methyl-2-thiophenecarbaldehyde		3209 2203 13679-70-4
15.024 1051	3-Acetyl-2,5-dimethylthiophene		3527 11603 2530-10-1



### 3. Additionally genotoxicity data submitted for subgroup 5.2

The Industry has submitted genotoxicity studies for one, 5-methyl-2-thiophenecarbaldehyde [FL-no: 15.004], of the two substances in this subgroup (EFFA, 2012). According to the Industry additional genotoxicity data for the other substance [FL-no: 15.024] will be submitted on a later stage.

The new data submitted for 5-methyl-2-thiophenecarbaldehyde [FL-no: 15.004] covers both *in vitro* and *in vivo* genotoxicity assays.

#### 3.1. *In vitro* data

*5-Methyl-2-thiophenecarbaldehyde [FL-no: 15.004]*

##### 3.1.1. Bacterial Reverse Mutation Assay

5-Methyl-2-thiophenecarbaldehyde was tested for the induction of gene mutations in the *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and TA102 both in the absence and in the presence of Aroclor induced rat liver S9-mix. Three independent experiments were performed (Beevers, 2009). An initial toxicity range finding experiment was carried out in the absence and presence of S9-mix in strain TA100. Six concentrations were tested in the concentration range 1.6 - 5000 µg/plate of 5-methyl-2-thiophenecarbaldehyde. Negative (solvent) and positive controls were included. Toxicity, evident as a decrease in revertant count, was apparent on all plates treated at 1000 µg/plate and above in the absence and presence of S9-mix, but revertant counts were obtained from at least four different concentrations, and these data were included as part of experiment 1.

In the first main experiment 5-methyl-2-thiophenecarbaldehyde was tested in the remaining 4 strains in the absence and presence of S9-mix using the plate incorporation methodology at concentrations ranging from 0.32 - 1000 µg/plate. Based on the range finding study the maximum tested concentration was reduced to 1000 µg/plate. Evidence of toxicity was observed at 200 µg/plate and above in strains TA1537 and TA102, in the presence of S9-mix, and at 1000 µg/plate in strains TA98 and TA102 in the absence of S9-mix and in strain TA98 and TA1535 in the presence of S9-mix. However, revertant counts were obtained from six different concentrations and so the data were considered valid for evaluation.

In a second experiment, treatments of all the tester strains were performed in the absence and presence of S9-mix. For each strain the highest tested concentration was based on toxicity in the first experiment and narrowed concentration ranges were employed. In addition, all treatments in the presence of S9-mix were further modified by the inclusion of a 1-hour pre-incubation step. Clear evidence of toxicity was observed in strains TA98 and TA102 following treatment at the maximum test concentration in both the absence and presence of S9-mix, and in the strain TA1537 following treatment at the maximum concentration in the presence of S9-mix. However, toxicity was not seen at the concentrations tested in TA100 and TA1535 in the presence and absence of S9-mix or in TA1537 in the absence of S9-mix, and therefore it was considered that higher concentrations should be evaluated. For the other strains data from a sufficient number of concentrations were obtained.

In the third experiment, 5-methyl-2-thiophenecarbaldehyde was tested in TA100 and TA1535 in the absence and presence of S9-mix and in TA1537 in the absence of S9-mix at 156.25 - 5000 µg/plate. Following these treatments, evidence of toxicity was observed at 2500 µg/plate and above in strains TA100 and TA1535 in the presence of S9-mix only.

No statistically significant increases in revertant numbers were observed in any of the tester strains that were both concentration-related and clearly reproducible. Some small increases in revertant numbers were observed in strain TA1535 in the absence of S9-mix, but these were sporadic, not concentration related and not reproducible. They were therefore considered to be chance occurrences and not a compound-related effect and therefore not biological relevant.



It was concluded that 5-methyl-2-thiophenecarbaldehyde did not induce mutation in five histidine-requiring strains (TA98, TA100, TA1535, TA1537 and TA102) of *S. typhimurium* when tested under the conditions of this study. These conditions included treatments at concentrations up to either the limit of toxicity or 5000 µg/plate (the maximum recommended concentration according to current regulatory guidelines), in both the absence and in the presence of a rat liver metabolic activation system (S9-mix).

The results of the *in vitro* studies are summarised in Table 4.

### 3.1.2. *In vitro* micronucleus assays

5-Methyl-2-thiophenecarbaldehyde was tested for the induction of chromosome damage and potential aneugenic effects in an *in vitro* micronucleus assay using duplicate human peripheral blood lymphocytes prepared from pooled blood from two healthy volunteers in two separate experiments. Treatments were performed both in the absence and presence of Aroclor 1254 induced rat liver S9-mix (Lloyd, 2011). Experiment 1 was conducted using blood from female donors and Experiment 2 was conducted using blood from male donors.

Treatment with 5-methyl-2-thiophenecarbaldehyde was conducted 48 hours after culture initiation (stimulation by phytohaemagglutinin).

A preliminary toxicity range finding experiment was conducted with S9-mix and 3 hours treatment and without S9-mix with 3 and 24 hours treatment. Toxicity was evaluated as the effect of treatment on the Replication Index (RI). Twelve concentrations from 4.6 to 1262 µg/mL were tested. The concentrations selected for the main experiments were based on toxicity data from this preliminary test.

In Experiment 1 (female donors) cells were exposed to 5-methyl-2-thiophenecarbaldehyde for 3 hours and 21 hours recovery (3 + 21) both with and without S9-mix. In addition, a continuous 24 hours treatment without recovery (24 + 0) was performed without S9 mix. All cultures were sampled 24 hours after the beginning of treatment (i.e. 72 hours after culture initiation). The concentrations selected for evaluation in the absence of S9-mix and 3 hours exposure were 600, 900 and 1000 µg/mL and in the presence of S9-mix and 3 hours exposure 50, 60 and 70 µg/mL. After 24 hours exposure cultures exposed to 120 µg/mL, 240 µg/mL, 300 µg/mL and 350 µg/mL were evaluated. Relevant positive and negative controls were included in all experiments. At the first test conditions (3 + 21 hours without S9-mix) no significant increases in the frequency of micronucleated binucleate cells (MNBN) were observed relative to concurrent vehicle controls at all concentrations analysed. Furthermore, the MNBN cell frequencies in all treated cultures under this treatment condition fell within the 95<sup>th</sup> percentile of the normal range.

In the 3 + 21 hours treatment condition with S9-mix the frequency of MNBN cells were significantly higher (1.05 %, 1.03 % and 1.33 % at 50, 60 and 70 µg/mL respectively) ( $p \leq 0.001$ ) than concurrent controls (0.31 %) at all concentrations analysed. The initial analysis of 1000 binucleate cells/culture revealed increased MNBN cell frequencies that exceeded the 95<sup>th</sup> percentile of the normal range for female donors in one of the two replicate cultures at 50 and 60 µg/mL and in both replicate cultures at 70 µg/mL. Following the additional analysis of 1000 binucleate cells/culture from the vehicle controls and the test concentrations, the frequencies of MNBN cells were still significantly higher ( $p \leq 0.001$ ) than those observed in concurrent controls at all three concentrations analysed. The MNBN cell frequencies in one replicate culture at 60 µg/mL and in both replicate cultures at 70 µg/mL (1.33 %) exceeded the 95<sup>th</sup> percentile of the normal range (0.1 - 1.2 %), however, both cultures at 50 µg/mL (1.05 %) fell within the normal range. These observations are indicative of a weak induction of micronuclei.

As a follow up of this positive result a second experiment was performed with lymphocytes from male donors to explore whether the weak induction of micronuclei that was observed in Experiment 1 in the presence of S9-mix could be due to the low MN frequencies in control cultures from female blood

donors. Following treatment for 3 hours in the presence of S9-mix with 5-methyl-2-thiophenecarbaldehyde at concentrations of 50 µg/mL, 60 µg/mL, 70 µg/mL and 80 µg/mL, followed by 21 hours recovery, and analysis of 1000 binucleate cells/culture, the frequencies of MNBN cells (0.9 %) were significantly higher ( $p \leq 0.001$ ) at 70 µg/mL compared to concurrent vehicle controls (0.30 %). The MNBN cell frequencies in single replicate cultures at 70 and 80 µg/mL exceeded the 95<sup>th</sup> percentile of the normal range for male donors (0.0 - 0.7 %) but the MNBN frequency at 80 µg/mL fell within the normal range. There was a concentration-dependent MN response from 50 – 70 µg/mL, with 70 µg/mL exceeding the normal range (0.90 %). An additional 1000 binucleate cells/culture were analysed, and as a result of the additional scoring the MNBN cell frequencies were significantly higher ( $p \leq 0.05$ ) than concurrent vehicle controls at the three highest concentrations analysed (60, 70, and 80 µg/mL). However, the cumulative MNBN cell frequencies exceeded the normal range at only the 70.00 µg/mL concentration (and attributable to only one of two cultures). These results are again indicative of weak induction of micronuclei.

In all of the different treatment conditions and separate experiments, negative control frequencies of MNBN were normal and were significantly increased by treatment with the positive control chemical.

In conclusion, 5-methyl-2-thiophenecarbaldehyde weakly induced micronuclei in both male and female human peripheral blood lymphocytes cultures when tested for 3 + 21 hours in the presence of S9-mix. In the same test system 5-methyl-2-thiophenecarbaldehyde did not induce micronuclei at up to toxic concentrations for 3 + 21 hours and 24 + 0 hours in the absence of S9-mix.

The results of the *in vitro* studies are summarised in Table 4.

### 3.2. *In vivo* data

#### 3.2.1. *In vivo* Combination Assay (Comet + Micronucleus)

On the basis of the *in vitro* micronucleus study reported above, as a next step to probe the genotoxic potential of 5-methyl-2-thiophenecarbaldehyde, a combined Comet assay and an *in vivo* micronucleus assay was carried out in rats (Beevers, 2012). This combined approach minimised the number of animals used in the experiments. Micronuclei were measured in bone marrow, but additionally, the liver was chosen as the most appropriate tissue for analysis in the Comet assay due to the fact that S9 metabolic activation was necessary to produce weakly positive results in the *in vitro* micronucleus assay, and this organ is the primary site of metabolism. Therefore, groups of Han Wistar male rats were administered 5-methyl-2-thiophenecarbaldehyde via gavage and the liver and bone marrow were analysed for the potential induction of DNA damage.

An initial dose range finding study was conducted to estimate the Maximum Tolerated Dose (MTD) of 5-methyl-2-thiophenecarbaldehyde after administration by oral gavage to groups of three male and three female Han Wistar rats. Doses of 1000 mg/kg bw/day resulted in mortality in both male and female rats while at 700 mg/kg bw/day mortality occurred in the female group but not in the male group. On this basis, 700 mg/kg bw/day was considered the MTD in males and 500 mg/kg bw/day was considered the MTD in females. Although there was a slight difference in MTD between males and females, it was less than 2-fold. Moreover, below 700 mg/kg bw/day no gender differences in clinical signs of toxicity were observed. It was therefore concluded that male rats alone could be used in the combined Comet and micronucleus assay.

Groups of six male Han Wistar rats were treated by oral gavage with 5-methyl-2-thiophenecarbaldehyde at doses of 70, 350 and 700 mg/kg bw/day, including a vehicle control (5 % w/v aqueous methylcellulose) and a positive control (ethyl methanesulphonate, 150 mg/kg bw/day). Animals were dosed at 0, 24 and 45 hours. Clinical signs of toxicity and body weight were recorded at each time point within the study. Three hours after the last dose (i.e. at 48 hours) the liver and one femur were removed from each control (negative and positive) and each treated animal for analysis of comets and micronuclei respectively. In a satellite group of animals (N = 3 per group) dosed similarly,

0.5 mL samples of blood were taken from the jugular vein at 0.5, 1, 2, 4, 8 and 24 hours after the final dose in case bioanalytical proof of exposure was subsequently needed.

No clinical signs of toxicity were observed for any animal in the treatment or control groups. No effect of treatment on body weight was observed. Clinical chemistry results did not present marked changes between treatment or control groups with two exceptions. Levels of aspartate aminotransferase were increased following dosing at 700 mg/kg bw/day compared to control values. Additionally, a histological observation of glycogen deposits in the liver of animals dosed at 350 and 700 mg/kg bw/day, along with changes in liver enzymes, indicate that the liver was exposed to the test article, 5-methyl-2-thiophenecarbaldehyde. These observations indicated exposure to the target organ (the liver) of the Comet assay (see below).

**In the micronucleus assay** femoral bone marrow was filtered through cellulose columns to remove the majority of nucleated cells, smears were made, fixed and stained with acridine orange. Two thousand polychromatic erythrocytes (PCE) per animal were scored for micronuclei under fluorescence microscopy. The data revealed that rats treated with 5-methyl-2-thiophenecarbaldehyde at all doses exhibited group mean % PCE (out of total erythrocytes) that were similar to the vehicle control group confirming that there was no evidence of test article-related bone marrow toxicity. Micronucleus frequencies in vehicle control rats were normal and were significantly increased by positive control treatment. Rats treated with 5-methyl-2-thiophenecarbaldehyde at all doses exhibited micronuclei PCE frequencies that were similar to the vehicle control group and which were considered consistent with the laboratory's historical data. There were no statistically significant increases in micronucleus frequency for any of the groups receiving the test article, compared to the concurrent vehicle control. There was no evidence of bone marrow toxicity, and therefore no direct evidence that the substance did reach the bone marrow. Therefore, no firm conclusion could be drawn on this part of the study.

**In the alkaline Comet assay**, liver samples from all control and test article treated animals were washed thoroughly, cut into small pieces in Merchants solution and then pushed through bolting cloth to produce single cell suspensions. Four slides were prepared per single cell suspension. Single cells were imbedded in agarose and once gelled, all slides were placed overnight in lysis buffer. Following lysis 3 of the 4 slides for each tissue and animal were transferred to electrophoresis buffer (pH >13) and the DNA unwound for 30 minutes and were electrophoresed in the same buffer at 0.7 V/cm for 40 minutes. After the lysis step, the 4<sup>th</sup> slide from each tissue and animal was placed in pH 7.0 buffer for approximately 3 x 5 minutes and then dried. This 'diffusion' slide was used to estimate the degree of damaged cells in the cell suspensions.

After staining with ethidium bromide tail moment and tail intensity (% DNA in tail) were obtained from 100 cells/animal/tissue (50 cells from each of two slides, where possible). Each slide was examined for possible indications of cytotoxicity. The number of 'clouds' out of 100 cells was scored for each slide. 'Clouds' were not used for comet analysis. Vehicle control animals exhibited quite low comet scores, but significant DNA damage was induced by the positive control. The Comet analysis revealed that animals treated with 5-methyl-2-thiophenecarbaldehyde exhibited elevated mean tail intensities and tail moments compared to concurrent vehicle control animals. However, the majority of animals, including the vehicle controls had tail intensity values below the laboratory's historical control range and the elevated mean level was due to only one animal in each group. Thus, the data generated for this assay is considered to fall within the normal level of variation for the assay. In addition, there was no indication of dose response relationship. Therefore it is considered that 5-methyl-2-thiophenecarbaldehyde does not induce DNA damage in the livers of rats when administered by oral gavage up to the MTD of 700 mg/kg bw/day.

The results of the *in vivo* studies are summarised in Table 5.

Although the Panel noted that the negative control values were extremely low (mean tail intensity of 0.07) the assay was found acceptable because the positive control (EMS) was clearly positive (mean

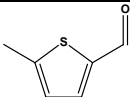
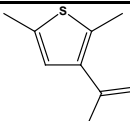
tail intensity of 29.43). The main problem with a low negative control value is that a test with low negative control values may have a difficulty to identify DNA crosslinking substances (with two reactive groups). However, the chemical structure of the test substance does not indicate a crosslinking potential. No studies on metabolism of [FL-no: 15.004] are available to the Panel. The CEF Panel recently (EFSA Scientific Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF), 2011) evaluated a structural related substance, 5-methyl furfural [FL-no: 13.001], in FGE.66Rev1 to have no concern for genotoxicity. The most likely metabolic conversions of 5-methyl furfural are oxidation of the aldehyde group to the carboxylic acid followed by conjugation with e.g. glycine or glucuronide, with rapid elimination in the urine. For furan and alkylfurans, ring opening has also been described, which would result in the formation of highly reactive unsaturated dialdehydes. In order to give an indication of whether ring opening could be possible for [FL-no: 15.004] an “evaluation” of the metabolism of this substance was run in a prediction programme (METEOR NEXUS version 1.5). In this programme no indications of ring opening were generated. Overall the Panel considered that the formation of a bifunctional DNA reactive metabolite is unlikely, and therefore concludes that this substance is not likely to have a cross-linking potential. The negative result of the Comet assay in the liver is considered acceptable.

## CONCLUSIONS

5-Methyl-2-thiophenecarbaldehyde [FL-no: 15.004] did not induce mutations in a gene mutation test in bacteria (Ames test). It did, however, induce weak genotoxic effects in an *in vitro* micronucleus assay in the presence of S9-mix. However, these weakly positive *in vitro* results were not confirmed in an *in vivo* combination assay (Comet assay in liver + micronucleus assay in bone marrow) in male rats when dosed up to the MTD. The Panel therefore concluded that 5-methyl-2-thiophenecarbaldehyde [FL-no: 15.004] does not give rise to concern with respect to genotoxicity and can accordingly be evaluated using the Procedure. For the other substance in subgroup 5.2, 3-acetyl-2,5-dimethylthiophene [FL-no: 15.024], the requested genotoxicity data are still pending and no conclusion could be drawn in the present FGE.

## SPECIFICATION SUMMARY OF THE SUBSTANCES IN THE FLAVOURING GROUP EVALUATION 224

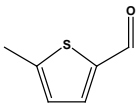
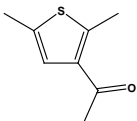
**Table 2:** Specification Summary of the Substances in the present group (JECFA, 2002b)

FL-no JECFA-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility 1) Solubility in ethanol 2)	Boiling point, °C 3) Melting point, °C ID test Assay minimum	Refrac. Index 4) Spec.gravity 5)
15.004 1050	5-Methyl-2-thiophenecarbaldehyde		3209 2203 13679-70-4	Liquid C <sub>6</sub> H <sub>6</sub> OS 126.18	Miscible	113-114 (33hPa)  NMR 95 %	1.574-1.586 1.168-1.172
15.024 1051	3-Acetyl-2,5-dimethylthiophene		3527 11603 2530-10-1	Liquid C <sub>8</sub> H <sub>10</sub> OS 154.23	Insoluble Miscible	105-108 (20hPa)  NMR 96 %	1.541-1.548 1.084-1.088

- 1) Solubility in water, if not otherwise stated.
- 2) Solubility in 95 % ethanol, if not otherwise stated.
- 3) At 1013.25 hPa, if not otherwise stated.
- 4) At 20°C, if not otherwise stated.
- 5) At 25°C, if not otherwise stated.

## CURRENT SAFETY EVALUATION STATUS APPLYING THE PROCEDURE (BASED ON INTAKES CALCULATED BY THE MSDI APPROACH)

**Table 3:** Summary of Safety Evaluation of the JECFA substances in the present group (JECFA, 2002a)

FL-no JECFA-no	EU Register name	Structural formula	EU MSDI 1) US MSDI ( $\mu\text{g/capita/day}$ )	Class 2) Evaluation procedure path 3)	JECFA Outcome on the named compound [4) or 5)]	EFSA conclusion on the named compound (genotoxicity)
15.004 1050	5-Methyl-2-thiophenecarbaldehyde		0.73 0.01	Class II B3: Intake below threshold, B4: Adequate NOAEL exists	4)	Evaluated in FGE.224, genotoxicity concern could be ruled out. Can be evaluated using the Procedure.
15.024 1051	3-Acetyl-2,5-dimethylthiophene		18 0.2	Class II B3: Intake below threshold, B4: Adequate NOAEL exists	4)	Evaluated in FGE.224, additional genotoxicity data required.

- 1) EU MSDI: Amount added to food as flavour in (kg / year) x 10E9 / (0.1 x population in Europe (= 375 x 10E6) x 0.6 x 365) =  $\mu\text{g/capita/day}$ .
- 2) Thresholds of concern: Class I = 1800  $\mu\text{g/person/day}$ , Class II = 540  $\mu\text{g/person/day}$ , Class III = 90  $\mu\text{g/person/day}$ .
- 3) Procedure path A substances can be predicted to be metabolised to innocuous products. Procedure path B substances cannot.
- 4) No safety concern based on intake calculated by the MSDI approach of the named compound.
- 5) Data must be available on the substance or closely related substances to perform a safety evaluation.

## GENOTOXICITY (IN VITRO)

**Table 4:** Summary of Additionally submitted *in vitro* genotoxicity data on [FL-no: 15.004] of subgroup 5.2 of FGE.19

FL-no	Chemical Name	Test System <i>in vitro</i>	Test Object	Concentrations of Substance and Test Conditions	Result	Reference	Comments
[15.004]	5-Methyl-2-thiophenecarbaldehyde	Reverse Mutation	<i>S. typhimurium</i> TA98, TA1535, TA1537 and TA102	0.32-1000 µg/plate [1,2];	Negative	(Beevers, 2009)	Valid study performed in accordance with OECD Guideline 471 and in compliance with GLP.
			<i>S. typhimurium</i> TA100	1.6-5000 µg/plate [1,2]			
			<i>S. typhimurium</i> TA98a, TA100b, TA102c, TA1535d, and TA1537e	10.24-1000 µg/plate [2,4,a,b,c]; 10.24-1000 µg/plate [3,5,a,d]; 25.6-2500 µg/plate [2,4,d,e]; 4.096-400 µg/plate [3,5,b,c,e]	Negative		
		Micronucleus Assay	<i>S. typhimurium</i> TA100a, TA1535b, TA1537c	156.25-5000 µg /plate [2,5,a,b]; 156.25-5000 µg /plate [2,4,a,b,c];	Negative	(Lloyd, 2011)	Valid study performed in accordance with OECD guideline 471 and in compliance with GLP.
			Human peripheral blood lymphocytes (Female and Male Donors)	600-1000 µg/ml [4,6]; 50-70 µg/mL [5,6]; 120-350 µg/mL [4,7]; 50-80 µg/mL [5,6]	Weak positive +S9.		

[1] With and without S9 metabolic activation.

[2] Plate incorporation method.

[3] Pre-incubation method.

[4] Without S9 metabolic activation.

[5] With S9 metabolic activation.

[6] 3-hour incubation with 21-hour recovery period.

[7] 24-hour incubation with no recovery period.



## GENOTOXICITY (IN VIVO)

**Table 5:** Summary of Additionally submitted *in vivo* genotoxicity data on [FL-no: 15.004] of subgroup 5.2 of FGE.19

FL-no	Chemical Name	Test System <i>in vivo</i>	Test Object / Administration	Concentrations of Substance and Test Conditions	Result	Reference	Comments
[15.004]	5-Methyl-2-thiophenecarbaldehyde	Micronucleus Assay in rat bone marrow	Han Wistar rats (F+M) / Gavage	70, 350, and 700 mg/kg bw/day (males only)	Negative	(Beevers, 2012)	Valid study.in accordanace with draft OECD Guideline 474 (2012). and in compliance with GLP Top dose was the maximum tolerated. Systemic exposure indicated by liver function changes.
		Comet assay in rat liver	Han Wistar rats (F+M) / Gavage	70, 350, and 700 mg/kg bw/day (males only)	Negative	(Beevers, 2012)	The study is in compliance with international accepted guidelines. and in compliance with GLP Top dose was maximum tolerated. Exposure to target organ indicated by liver function changes.

## REFERENCES

- Beevers C, 2009. Reverse mutation in five histidine-requiring strains of *Salmonella typhimurium*. 5-methyl-2-thiophenecarbaldehyde. Covance Laboratories Ltd. Study no. 8201127. November 2009. Unpublished report submitted by EFA to FLAVIS Secretariat.
- Beevers C, 2012. Combined Comet assay in the liver and a bone marrow micronucleus test in treated rats. 5-Methyl-2-thiophenecarbaldehyde. Covance Laboratories Ltd. Study no. 8250473. June 2012. Unpublished report submitted by EFA to FLAVIS Secretariat.
- Benigni R and Netzeva T, 2007a. Report on a QSAR model for prediction of genotoxicity of alpha,beta-unsaturated aldehydes in *S. typhimurium* TA100 and its application for predictions on alpha,beta-unsaturated aldehydes in Flavouring Group Evaluation 19 (FGE.19). Unpublished report submitted by FLAVIS Secretariat to EFSA.
- Benigni R and Netzeva T, 2007b. Report on a QSAR model for prediction of genotoxicity of alpha,beta-unsaturated ketones in *S. typhimurium* TA100 and its application for predictions on alpha,beta-unsaturated aldehydes in Flavouring Group Evaluation 19 (FGE.19). Unpublished report submitted by FLAVIS Secretariat to EFSA.
- EC, 1996. Regulation No 2232/96 of the European Parliament and of the Council of 28 October 1996. Official Journal of the European Communities 23.11.1996, L 299, 1-4.
- EC, 1999. Commission Decision 1999/217/EC of 23 February 1999 adopting a register of flavouring substances used in or on foodstuffs. Official Journal of the European Communities 27.3.1999, L 84, 1-137.
- EC, 2000. Commission Regulation No 1565/2000 of 18 July 2000 laying down the measures necessary for the adoption of an evaluation programme in application of Regulation (EC) No 2232/96. Official Journal of the European Communities 19.7.2000, L 180, 8-16.
- EC, 2002. Commission Regulation No 622/2002 of 11 April 2002 establishing deadlines for the submission of information for the evaluation of chemically defined flavouring substances used in or on foodstuffs. Official Journal of the European Communities 12.4.2002, L 95, 10-11.
- EC, 2009. Commission Decision 2009/163/EC of 26 February 2009 amending Decision 1999/217/EC as regards the Register of flavouring substances used in or on foodstuffs. Official Journal of the European Union 27.2.2009, L 55, 41.
- EC, 2012. Commission implementing Regulation (EU) No 872/2012 of 1 October 2012 adopting the list of flavouring substances provided for by Regulation (EC) No 2232/96 of the European Parliament and of the Council, introducing it in Annex I to Regulation (EC) No 1334/2008 of the European Parliament and of the Council and repealing Commission Regulation (EC) No 1565/2000 and Commission Decision 1999/217/EC. Official Journal of the European Communities 2.10.2012, L 267, 1-161.
- EFA, 2012. Submission by the European Flavour Association to the European Food Safety Authority. Flavouring Group Evaluation 19 Subgroup 5.2 Flavouring Substance of the Chemical Group 3 (Annex I of 1565/2000/EC) Pyrroles, Thiophenes and Other Sulphur-containing Substances Used as Flavouring Substances. May 2012. FLAVIS/8.157.
- EFSA, 2008a. Minutes of the 26<sup>th</sup> Plenary meeting of the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food, Held in Parma on 27 - 29 November 2007. Parma, 7 January 2008. [Online]. Available: [http://www.efsa.europa.eu/EFSA/Event\\_Meeting/afc\\_minutes\\_26thplen\\_en.pdf](http://www.efsa.europa.eu/EFSA/Event_Meeting/afc_minutes_26thplen_en.pdf)
- EFSA, 2008b. Genotoxicity Test Strategy for Substances belonging to Subgroups of FGE.19 - Statement of the Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF). EFSA Journal (2008) 854, 1-5. [Online]. Available: [http://www.efsa.europa.eu/EFSA/efsa\\_locale-1178620753812\\_1211902211354.htm](http://www.efsa.europa.eu/EFSA/efsa_locale-1178620753812_1211902211354.htm)

- EFSA, 2008c. Scientific opinion. List of alpha,beta-unsaturated aldehydes and ketones representative of FGE.19 substances for genotoxicity testing. Statement of the Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF). Adopted on 27 November 2008, updated 26 March 2009. EFSA-Q-2008-709.
- EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF), 2011. Scientific Opinion on Flavouring Group Evaluation 66, Revision 1 (FGE.66Rev1): Consideration of Furfuryl Alcohol and Related Flavouring Substances Evaluated by JECFA (55<sup>th</sup> meeting). EFSA Journal 2011;9(9):2314. [44 pp.]. doi:10.2903/j.efsa.2011.2314. Available online: [www.efsa.europa.eu/efsajournal.htm](http://www.efsa.europa.eu/efsajournal.htm)
- Gry J, Beltoft V, Benigni R, Binderup M-L, Carere A, Engel K-H, Gürtler R, Jensen GE, Hulzebos E, Larsen JC, Mennes W, Netzeva T, Niemelä J, Nikolov N, Nørby KK and Wedebye EB, 2007. Description and validation of QSAR genotoxicity models for use in evaluation of flavouring substances in Flavouring Group Evaluation 19 (FGE.19) on 360 alpha,beta-unsaturated aldehydes and ketones and precursors for these. Unpublished report submitted by FLAVIS Secretariat to EFSA.
- JECFA, 2002a. Evaluation of certain food additives. Fifty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives. WHO Technical Report Series, no. 913. Geneva, 4-13 June 2002.
- JECFA, 2002b. Compendium of food additive specifications. Addendum 10. Joint FAO/WHO Expert Committee of Food Additives 59<sup>th</sup> session. Geneva, 4-13 June 2002. FAO Food and Nutrition paper 52 Addition 10.
- JECFA, 2003. Safety evaluation of certain food additives. Fifty-ninth Meeting of the Joint FAO/WHO Expert Committee on Food Additives, WHO Food Additives Series: 50. IPCS, WHO, Geneva
- Lloyd M, 2011. Induction of micronuclei in cultured human peripheral blood lymphocytes. 5-Methyl-2-thiophenecarbaldehyde. Covance Laboratories LTD. Study no. 8218058. June 2011. Unpublished report submitted by ECHA to FLAVIS Secretariat.
- Nikolov N, Jensen GE, Wedebye EB and Niemelä J, 2007. Report on QSAR predictions of 222 alpha,beta-unsaturated aldehydes and ketones from Flavouring Group Evaluation 19 (FGE.19) on 360 alpha,beta-unsaturated aldehydes and ketones and precursors for these. Unpublished report submitted by FLAVIS Secretariat to EFSA.
- SCF, 1999. Opinion on a programme for the evaluation of flavouring substances (expressed on 2 December 1999). Scientific Committee on Food. SCF/CS/FLAV/TASK/11 Final 6/12/1999. Annex I the minutes of the 119<sup>th</sup> Plenary meeting. European Commission, Health & Consumer Protection Directorate-General.

## ABBREVIATIONS

AFC	Food Additives, Flavourings, Processing Aids and Materials in Contact with Food
CAS	Chemical Abstract Service
CEF	Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
CoE	Council of Europe
DNA	Deoxyribonucleic acid
EFSA	The European Food Safety Authority
EU	European Union
FGE	Flavouring Group Evaluation
FLAVIS (FL)	Flavour Information System (database)
GLP	Good Laboratory Practice
ID	Identity
IR	Infrared spectroscopy
JECFA	The Joint FAO/WHO Expert Committee on Food Additives
LMA	Low Melting point Agarose
MNBN	MicroNucleated BiNucleate cells
MS	Mass spectra
MTD	Maximum Tolerated Dose
NMA	Normal Melting point Agarose
NMR	Nuclear Magnetic Resonance
No	Number
OECD	Organisation for Economic Co-operation and Development
PCE	Polychromatic Erythrocytes
(Q)SAR	(Quantitative ) Structure Activity Relationship
RI	Replication Index
SCF	Scientific Committee on Food
WHO	World Health Organisation